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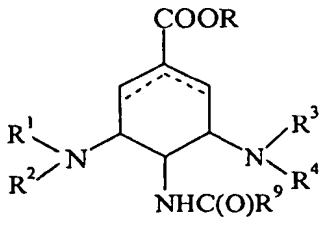
(54) Title: A METHOD OF FORMING NEURAMINIDASE INHIBITORS BY DYNAMIC COMBINATORIAL CHEMISTRY AND COMPOUNDS OBTAINED



(I)

(57) Abstract: The present invention relates to compounds according to the formula (I), wherein the dotted line denotes a double bond which is present in one of the two possible positions; R^1 to R^4 are independently of each other selected from the group consisting of: hydrogen, C_1-C_{20} alkyl groups, C_2-C_{20}-alkenyl groups, C_4-C_{20} aryl groups, C_5-C_{20}-aralkyl groups and C_5-C_{20}-alkaryl groups, all of which groups all can contain one or more hetero atoms from the group consisting of N, O, and S, and which groups can carry one or more substituents from the group consisting of hydroxyl groups and C_1-C_4-alkyl ester groups; or one of the substituents NR^1R^2 and NR^3R^4 is a guanidino group of the formula NR^5-$C(NR^6R^7)$=NR^8 in which the substituents R^5 to R^8 independently of each other have the meaning given above for the substituents R^1 to R^4; R^9 is a C_1-C_4-alkyl group, or a physiologically acceptable salt or solvate thereof in any stereoisomenc form or mixtures thereof in any ratio. A further object of the invention is a method of forming a library of components which are potentially capable of binding to neuraminidase, in particular influenza neuraminidase, which method comprises i) selecting a plurality of molecules carrying a functionality which may interact with a binding site of neuraminidase, said molecules furthermore having a linking group which is capable of interacting with other linking groups under the formation of reversible bonds; ii) reacting the molecules carrying the functionality with a molecule according to formula (I) as defined in claim 1 in the presence of the target, under conditions where a formation of reversible bonds between the linking groups on the molecule (I) and on the molecules carrying a functionality occurs.

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A method of forming neuraminidase inhibitors by dynamic combinatorial chemistry and compounds obtained

The present invention relates to dynamic combinatorial chemistry (DCC), more particularly to the use of DCC to identify compounds which are active as neuraminidase inhibitors.

New chemical or biological entities with useful properties are classically generated by identifying a chemical or biological compound (a so-called lead compound) with some desirable properties or activities, creating varieties of said compound to form a library, and evaluating the properties and activities of those variant compounds.

The conventional approach is limited by the relatively small pool of previously identified compounds which may be screened to identify new compounds with the desirable property or activity.

Another drawback pertains to the step of the creation of variants. Traditionally, compound variants are generated by chemists or biologists using a conventional chemical or biological synthesis procedure. Thus, the generation of compound variants is time-consuming and requires huge amounts of work.

To assist in the generation of new chemical compounds, attention has recently turned to the use of combinatorial chemical libraries.

Combinatorial chemistry (CC) has experienced an explosive growth in recent years. It provides a powerful methology for exploring the molecular geometrical and interactional

spaces through molecular diversity generation. This is in particular the case for the discovery of new biologically active substances and medical drugs. It resides on the constitution of vast combinatorial libraries (CLs), extensive collections of molecules derived from a set of units connected by successive and repetetive application of specific chemical reactions. It is thus based on a large population of different molecules that are present as discrete entities.

The constitution of a CL of components amounts to the fabrication of a large collection of components. The CL is then screened against a target, with the goal that one of its constituents will fit the target lock/receptor, i.e. show an activity, and be retrievable from the mixture.

In contrast to this, the present invention makes use of the so-called dynamic combinatorial chemistry which is a conceptionally different approach. It relies on a reversible connection process for the spontaneous and continuous generation of all possible combinations of a set of basic constituents, thus making virtually available all structural and interactional features that these combinations may present. Such multicomponent self-assembly amounts to the presentation of a dynamic combinatorial library (DCL) which is a potential library made up of all possible combinations in number and nature of the available components. By recruiting the correct partners from the set of those available, the component, among all those possible, that possesses the features most suitable for formation of the optimal supramolecular entity with the target site is selected. The composition of the set of components/subunits depends on the extent to which the possible combinations cover the geometrical and interactional spaces of the targets.

Self-assembly in a multi-component system is a combinatorial process with a search procedure directed by the kinetic and thermodynamic parameters imposed by the nature of constituents and their interactions.

In WO 97/43232 there is disclosed a substance library which library consists of molecular species which are bonded to a molecular pairing system. The pairing system is composed of molecules, in the preferred embodiment, which are selected amongst specially designed

nucleic acids which can bind to each other in a certain manner which results in a particular geometric form. The molecular species are selected, in a preferred embodiment, from the group consisting of peptides, and these peptides are designed according to the particular requirements of a given component which is brought into contact with the library component. The complex which forms upon contact with the component is identified, in order to evaluate the interaction between a component and complex.

In Proc. Natl. Acad. Sci. USA 1997 (94), 2106, Ivan Huc and Jean-Marie Lehn disclose a method for the generation of a dynamic combinatorial library of imines from structural fragments bearing aldehyde and amino groups. The method is directed toward the synthesis of inhibitors of the enzyme carbonic anhydrase by recognition-involved assembly, and the synthesis of the above-mentioned imines is carried out in the presence of the said enzyme carbonic anhydrase. It was found that reversible combination of the used amines and aldehydes leads to the shift of the equilibrium population towards the imine product that was closest in structure to a known highly efficient inhibitor of the enzyme.

The application WO 01/64605 discloses a process for establishing a dynamic combinatorial library for a target which binds at least two functionalities. The method comprises selecting a plurality of molecules carrying functionalities which, by combination with each other, are capable of forming an entity which may bind to the functionalities in the target. The molecules carrying the functionalities are linked by a spacer group which allows a reversible formation and cleavage of bonds. The cleavage and formation of the bonds can occur in the spacer group, or at the terminus of it. Generally, the spacer group will be selected such as to potentially fit into the binding site(s) of the target. The spacer groups disclosed in the application are all linear entities like for example alkane derivatives, and contain a maximum of two linking groups allowing the formation and cleavage reversible bonds.

The co-pending application having the title "A method of forming a dynamic combinatorial library using a scaffold" of the applicant is drawn towards DCC methods which use scaffolds in the generation of the DCL. This method permits to generate libraries which contain complex molecules carrying numerous functionalities.

Neuraminidase is a major target for drug action on influenza. The enzyme structure is well characterized and multiple SAR data on the active site ligands are available in the literature. It has been shown that the presence and particular spatial arrangement of a number of linking groups in the neuraminidase ligands are crucial and conserved in a majority of known inhibitors. As a target, for example the influenza A virus neuraminidase is appropiate, a viral surface enzyme that catalyzes the cleavage of sialic acid residues terminally linked to glycoproteins and glycolipids and plays an important role in the propagation of the virus.

A class of compounds being neuraminidase inhibitors and active against the propagation of the virus is composed of cyclohexene carboxylic acid derivatives. This class of compounds is the object of several patent applications.

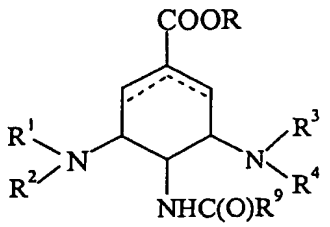
The application WO 99/31047 discloses cyclohexene carboxylate derivatives which are active as neuraminidase inhibitors. The compounds carry two amino substituents in 3- and 5-position and a $-NHCOO^-$ substituent in 4-position.

The application WO 01/28981 also discloses neuraminidase inhibitors which are based on cyclohexene carboxylic acid derivatives carrying amino groups in 3- and 5-position. The carbon atom in 3- or 5-position however carries, besides the amino substituent, a further substitutent which is not a hydrogen atom.

Further neuraminidase inhibitors which are based on cyclohexene carboxylic acid derivatives are disclosed in WO 98/07685, US 6,057,459, US 6,204,398 and US 5,859,284. The molecules disclosed in these documents all carry an alkoxy substituent.

The object underlying the present invention is to provide further neuraminidase inhibitors.

This object is obtained by the compounds according to formula I



I

wherein
the dotted line denotes a double bond which is present in one of the two possible positions;
R is hydrogen or a C_1-C_4 alkyl group;
R^1 to R^4 are independently of each other selected from the group consisting of:
hydrogen, C_1-C_{20} alkyl groups, C_2-C_{20}-alkenyl groups, C_4-C_{20} aryl groups, C_5-C_{20}-aralkyl groups and C_5-C_{20}-alkaryl groups, all of which groups can contain one or more hetero atoms from the group consisting of N, O, and S, and which groups can carry one or more substituents from the group consisting of hydroxyl groups and C_1-C_4-alkyl ester groups; or
one of the substituents NR^1R^2 and NR^3R^4 is a guanidino group of the formula $-NR^5-C(NR^6R^7)=NR^8$ in which the substituents R^5 to R^8 independently of each other have the meaning given above for the substituents R^1 to R^4;

R^9 is a C_1-C_4-alkyl group,
or a physiologically acceptable salt or solvate thereof in any stereoisomeric form or mixtures thereof in any ratio,
with the proviso that the compounds in which
R^1 is H, R^2 is $CH(CH_2CH_3)_2$, R^3, R^4 are H and R is ethyl and
R^1 is H, R^2 is $-CH_2CH_2CH_3$, R^3, R^4 are H and R is ethyl
are excluded.

The compounds of formula I are novel compounds and are an object of the present invention.

According to a embodiment of the present invention, the compounds according to formula I are used as a scaffold for the finding of neuraminidase inhibitors by a DCC-process.

The library of potential inhibitors can be designed in such a way that the components will be formed from a scaffold according to formula I having a basic affinity for the target active site. The scaffold can, for example, react with molecules carrying an aldehyde linking group or molecules carrying a keto group. In case ketones are used, higher concentrations of the compounds are required, in general. In the dynamic mixture, each of the amino groups in the

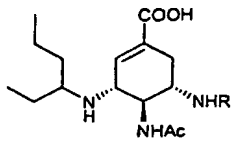
scaffold is in principle capable of reacting with any of the aldehyde groups to form a set of transient species **A** to **D** existing in a rapid dynamic equilibrium with each other and with the building blocks. Subsequent chemical reduction of the transient compounds with an externally added reagent irreversibly converts them a the set of amines **E** to **H** giving rise to a large array of stable library components, each containing up to four substituents attached to the scaffold (see Example 2 and Scheme 2). The presence of all mono-, di-, tri- and most of the tetrasubstituted components which can theoretically be formed can in many cases be proved experimentally, with the ratios changing over the course of the reaction.

Preferred compounds according to the formula I which can be used as a scaffold and/or a neuraminidase inhibitor are those in which both of the groups NR^1R^2 and NR^3R^4 are $-NH_2$ groups and those in which one of these groups is an $-NH_2$ group and the other is a guanidinium group of the formula $-NH-C(NH_2)=NH$.

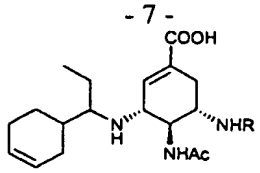
In a process as described above, the compounds **1** to **13** showing a neuraminidase inhibiting activity could be identified (see below).

According to a further embodiment of the invention the compounds according to formula I are active neuraminidase inhibitors. Some of these compounds have been identified by a dynamic screening process using a scaffold according to the general formula I. Examples of preferred compound of the formula I are those in which $R^9 = CH_3$, one of the groups NR^1R^2 and NR^3R^4 being $-NH-C(NH_2)=NH$ and the other group being $-NH_2$ which is substituted by an alkyl group which is optionally substituted by a 4- to 6-membered carbocycle optionally carrying 1 to 3 olefinic bonds. These compounds can be synthesized by using method known as such, for example by reductive amination of the respective aldehydes or ketones using the compound followed by hydrolysing the ester group and removing the protective groups, generally by hydrolysis.

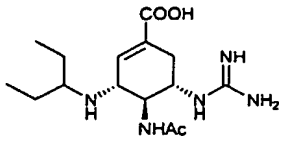
Particularly preferred compounds are those according to the formulae **9** to **13** in which Ac is $-C(O)CH_3$.



9 R = H
10 R = C(=NH)NH_2

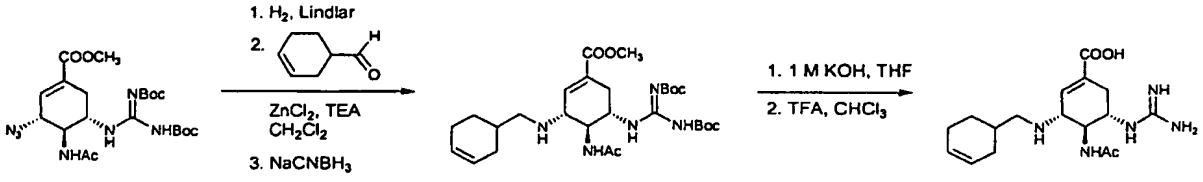


11 R = H
12 R = C(=NH)NH_2



13

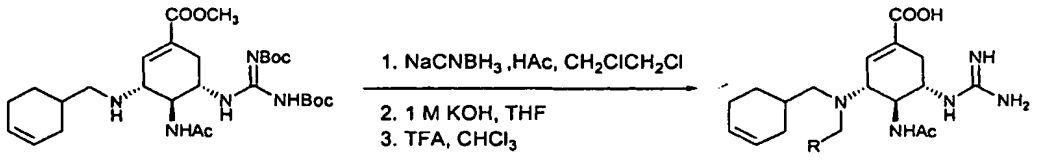
The most preferred compound is **7**, which inhibits neuraminidase with an efficiency similar to or even better than the known influenza drug oseltamivir.



V **Y** **7**

The compound is synthesized according to the above scheme. Another preferred compound is **8** (see Fig 5) which is synthesized in a way analogous to the above scheme.

Still a further object of the present invention are compounds according to formula I in which in which one of the groups $-NR^1R^2$ and $-NR^3R^4$ is a guanidinium group and the other group is an amino group which is not substituted by hydrogen. Within this group, the compounds **14** and **15** which can be synthesized as depicted in the following scheme are preferred.



Y

14 R = **Cyclopropyl**
15 R = **Ethyl**

Although the compounds according to the formulae **1** to **15** are shown in a specific stereoisomeric form, the present invention is not limited thereon and includes all stereoisomeric forms and mixtures thereof in any ratio.

A further object of the present invention are medicaments which contain an effective amount of a compound according to the formula I as defined above. This preferrably applies to the molecules which have above been denoted as being preferred compound classes, in particular to the compounds **7** to **15**. The compounds according to formula I are hence appropriate for the use as a medicament, preferably for inhibiting the activity of neuraminidase, in particular for inhibiting the activity of influenza neuraminidase. Still a further object is the treatment of a mammal, in particular a human, with a compound according to formula I, preferrably against the action of neuraminidase. The compounds can be used in any appropriate form, e.g. as such, in form of a physiologically acceptable salt or a physiologically active solvate. Further objects of the present invention are pharmaceutical preparations (or pharmaceutical compositions) which comprise an effective dose of a compound of the formula I and a pharmaceutically acceptable carrier, i.e. one or more pharmaceutically acceptable carrier substances and/or additives.

The compounds according to the formula I can also be used in combination with other pharmaceutically active compounds, preferably compounds which are able to enhance the effect of the compounds according to the formula I.

The compounds I, optionally in combination with other pharmaceutically active compounds, can be administered to animals, preferably to mammals, and in particular to humans, as pharmaceutical by itself, or in the form of pharmaceutical preparations.

The pharmaceutical according to the invention can be administered orally, for example in the form of pills, tablets, lacquered tablets, sugar-coated tablets, granules, hard and soft gelatin capsules, aqueous, alcoholic or oily solutions, syrups, emulsions or suspensions, or rectally, for example in the form of suppositories. Administration can also be carried out parenterally, for example subcutaneously, intramuscularly or intravenously in the form of solutions for injection or infusion. Other suitable administration forms are, for example, percutaneous or topical administration, for example in the form of ointments, tinctures, sprays or transdermal therapeutic systems, or the inhalative administration in the form of nasal sprays or aerosol mixtures, or, for example, microcapsules, implants or rods. The preferred administration form depends, for example, on the disease to be treated and on its severity.

The present invention will now be illustrated in the following non-limiting examples.

EXAMPLES

Example 1

Synthesis of the cyclic scaffolds **1** and **2**

The synthesis is laid out in the Scheme 1

Example 2

Generation of a dynamic combinatorial library with the scaffold 1

Neuraminidase is a major target for drug action on influenza. The enzyme structure is well characterized and multiple SAR data on the active site ligands are available in the literature. It has been shown that the presence and particular spatial arrangement of a number of linking groups in the neuraminidase ligands are crucial and conserved in a majority of known inhibitors. As a target, the influenza A virus neuraminidase was used, a viral surface enzyme that catalyzes the cleavage of sialic acid residues terminally linked to glycoproteins and glycolipids and plays an important role in the propagation of the virus. For solubility reasons, neuraminidase was expressed and the extra-cellular domain containing the active site was purified, see below. We designed the library of potential inhibitors in such a way that the components will be formed from a scaffold **1**. **1** contains a moiety similar to known influenza drugs and therefore possesses a basic affinity for the target active site. **1** reacts with molecules carrying an aldehyde linking group (Scheme 2). In the dynamic mixture, each of the amino groups in the scaffold is in principle capable of reacting with any of the aldehyde groups to form a set of transient species **A** to **D** existing in a rapid dynamic equilibrium with each other and with the building blocks. Subsequent chemical reduction of the transient hemiaminals and imines with an externally added reagent irreversibly converts them into the set of amines **E** to **H** giving rise to a large array of stable library components, each containing up to four substituents attached to the scaffold. The presence of all mono-, di-, tri- and most of the tetrasubstituted components which can theoretically be formed could be proved experimentally. Their ratios changed over the course of the reaction.

The addition of the target enzyme to the dynamic library shifts the equilibrium between the transient species toward the components with the highest affinity for the target. Chemical reduction of the library, after re-equilibriation in the presence of the target, should result in

the distribution of stable components reflecting the enrichment in the transient best binders. However, the analysis of such a mixture, even of enriched composition, and identification of the active components becomes extremely difficult with increasing size of the library. The reaction was thus carried out under conditions where the transient components were present only in trace amounts and the reduction reaction was sufficiently slow as not to produce significant amounts of any reduced species within a certain time laps. In such a virtual library, components cannot be detected, unless the presence of a target promotes the formation of preferred structures, due to the equilibrium shift and/or to the acceleration of irreversible coupling.

The substituents were initially grouped in two sets. The first set contained 10 aldehydes, some of which showed statistically significant inhibition of neuraminidase activity in the mixture with the scaffold in a preliminary screening. The second set included the aldehydes A4, A11, A14, A17, A18 that did not show inhibitory activity in the preliminary screen. The aldehydes employed are depicted in Fig. 1.

Brief incubation of the first scaffold-substituent mixtures with the enzyme, followed by reduction resulted in a striking amplification effect of selected library components, as seen from the HPLC-MS analysis, Fig. 2. The main type of species detected in the library was the monosubstituted scaffold. The concentrations of most library components in the absence of the enzyme were selected as to be below detectable level. In the presence of the target, the amplification of the adduct with aldehyde A22 was quantified and amounted to a factor of 120. No products were detected in the experiment with the second set of aldehydes. Notably, the substituents identified in the target-promoted subset of components also showed up in the preliminary activity screen. In both sets, the progress of the reaction was also controlled by the consumption of the scaffold, which in the first set was completely converted to the selected products, and in the second set was not consumed.

Kinetic studies of the product evolution in the presence of the target were conducted. In the experiment described above, the scaffold was consumed and the concentration of products leveled off within 10 h of reduction. No significant change in the mixture composition was observed for the following 48 h of incubation in the presence of the target. A control

experiment, without the target, showed slow accumulation of multiple coupling products over prolonged periods of time.

To see whether removal of the most effective substituent will change the library bias, an experiment was performed in which A22 in the first set was replaced by a randomly chosen aldehyde A26. The resulting analytical profile showed both secondary hits observed previously (A8 and A13), as well as another minor component formed from A4, see Fig. 3.

The diversity of the libraries were then increased and two larger sets of substituents were screened, one that included 20 aldehydes of purely aliphatic nature (Fig. A4), and another with 17 aldehydes containing aromatic groups (Fig. 4B). Both sets yielded the hits found in the smaller libraries and a new component formed from A39.

The potential diversity of the virtual libraries is very high, for example the set of 20 aldehydes potentially yields over 40.000 stable components. However, because of the intrinsic properties of the target, the library is biased toward formation of the monosubstituted components.

By synthesizing several representative library members as individual compounds and testing them in independent enzyme assays, it could be shown that the virtual libraries presented to the target indeed selectively generate components possessing higher inhibitory activity. Interestingly, even less effective substituent displays K_i significantly lower than the pure scaffold. The K_i values of some compounds are shown in Fig. 5

Experimental details:

A typical DCC experiment was carried out as follows. Aldehyde stock solutions were prepared by dissolving in DMSO in 1M concentration and dilution with with a 10 mM aqueous imidazole buffer, pH 7.8 to 10 mM. To a 10 μM solution of Neuraminidase (50 μl) in the imidazole buffer was added the scaffold (2 μl of 250 μM solution in the imidazole buffer pH 7.8) and premixed stock solutions of aldehydes, 1 μl each. The reaction mixture was incubated for 10 min and then tetrabutylammonium cyano borhydride (TBC) (2 μl of 2.5 mM solution in acetonitrile) was added. After incubation for 12h at 25°C the reaction mixture was analyzed by HPLC-MS.

HPLC-MS analyses were performed with electrospray ionization (positive mode) on a Bruker Esquire 3000 ion trap mass spectrometer connected to an Agilent 1100 HPLC. A gradient of 0.1 % formic acid in H_2O (A) : acetonitrile (B) was applied using a Phenomenex LUNA C18(2) 5µ reversed phase HPLC column (250 x 3.00 mm, flow rate 0.5 ml/min). Eluent composition was kept isocratic at 0 % B for 5 min. Subsequently, B was linearly increased in two steps to 20 % (t = 7 min) and to 50 % (t = 15 min) and then kept iscocratic at 50 % B for 5 min. MS data were acquired in the full scan mode and scanned for protonated molecular ions $[M^+H]^+$ of potential products in extracted ion chromatograms. Corresponding peak areas plotted in Figures 1-5 are proportional to the concentration of each component, but generally cannot be used to compare concentrations of two or more different components.

The neuraminidase activity was determined using the synthetic substrate 2'-(4-methylumbelliferyl)-a-D-N-acetylneuraminic acid (MU-NANA). The enzyme was incubated for 30 min at 37°C with 50 µM MU-NANA in 100 mM sodium acetate pH 5.5, 2 mM CaCl2 and subsequently the reaction was stopped with 200mM glycine pH10.5, 120mM NaCl for measuring the fluorescence of released 4-methylumbelliferone (4-MU) at an excitation wavelength of 355nm and an emission of 460nm. For neuraminidase activity testing we either used purified tNA-His protein originating of the avian influenza A/FPV/Rostock/34 (H7N1) strain (see above) or virus preparations of different human Influenza A [A/Panama /2007/99 (H3N2); A/Johannesburg/33/94 (H3N2)] and Influenza B (B/Harbin/7/94; B/Victoria/504/2000) strains.

The neuraminidase cDNA of the Influenza A/FPV/Rostock/34 was amplified and modified by PCR (forward primer: GGGGACAAGTTTGTACAAAAAAGCAGGCTGCCACCATGAA TCCAAATCAGAAAATAATAACC; reverse primer: GGGGACCACTTTGTACAAGAAAGCTGGGTTT ACTAGTGATGGTGATGGTGATGCGATCCCTTGTCAATGGTGAATGGCAACTCAGC) to give pDEST8-tNA-His which encodes for a neuraminidase with six histidines fused to the C-terminus (tNA-His).

Spodoptera frugiperda Sf-9 insect cells were cultivated at 27°C in the serum-free medium ExCell400 (JRH Bioscience). Exponentially growing cells (2 x 106 cells/ml) were infected

with baculovirus at a multiplicity of infection (MOI) of 10. After 72h of expression the cells were harvested and the extra-cellular domain of the neuraminidase (sol-tNA-His) was released by treatment with pronase (Ref. ...). Briefly, cells were treated for 2h at 37°C with pronase (1 mg/ml; CalBiochem) and DNaseI (50 µg/ml) in 100 mM sodium acetate pH 5.5, 2 mM $CaCl_2$, 10 mM $MgCl_2$. After separation cellular material and inactivation of pronase, sol-tNA-His was purified by metal chelate affinity chromatography using Ni-NTA superflow beads (Qiagen). The purification yielded an average of 3mg of sol-tNA-His out of 1l of culture, with a purity of >80% and a specific activity of ~4000 U/mg.

Example 3

Generation of a dynamic combinatorial library with scaffold **2**

In a further experiment, the structure of the scaffold was modified by replacing the amino group which in the active components had not been substituted with a guanidinium group, see compound **2**. Such a replacement yields better binding of the known inhibitors, as can be seen from the K_i value of **5** (Fig. 5). The experiments with the virtual libraries were performed similarly to those described in Example 2, and the subset of substitutes selected with the scaffold **2** was identical to that with **1**. The preferred components, **7** and **8** were also synthesized individually and showed K_i values of 16 and 52 nM, respectively. The identity of the individually synthesized stable components with the ones formed in the presence of the enzyme was confirmed by the HPLC-MS analysis.

The experimental details are the same as those given in Example 2.

Example 4

Generation of a dynamic combinatorial library using ketones

Ketone stock solutions were prepared by dissolving in DMSO in 1M or 5M concentration. To a 43 µM solution of Neuraminidase (50µl) in imidazole buffer (pH 7.8) was added the scaffold **1** or **2** (5 µl of a 1 mM solution in the imidazol buffer pH 7.8) and premixed stock solutions of ketones (0.25 µl of 5M solution in DMSO each). The reaction mixture was incubated for 10 min. and then tetrabutylammonium cyano borhydride (TBC) (4 µl of 0.1 mM

solution in acetonitril) was added. After incubation at 25°C the reaction mixture was analyzed by HPLC/MS.

HPLC-MS analyses were performed with electrospray ionization (positive mode) on a Micromass Quattro Ultima quadrupole-hexapole-quadrupole mass spectrometer connected to a Waters alliance™ 2695 HPLC. A gradient of 0.1 % formic acid in H_2O (A) : acetonitrile (B) was applied using a Phenomenex LUNA C18(2) 5µ reversed phase HPLC column (150 x 3.00 mm, flow rate 0.5 ml/min). Eluent composition was kept isocratic at 0 % B for 3 min. Subsequently, B was linearly increased in three steps to 50 % (t = 15 min), 90 % (t = 20 min), and to 95 % (t = 22 min), and finally decreased to initial conditions within 5 min. Re-equilibration time was 3 min. MS data were acquired in the selective reaction monitoring (SRM) mode and scanned for specific ion transitions of potential products. Suitable ion transitions for SRM measurements were determined by flow injection and MS/MS analysis (recording of product ion spectra) of standard compounds.

Example 5

In vitro activity of synthesized compounds (from aldehydes)

See Fig 5 for the formulae of the compounds mentioned in the following table.

Table 1. *Ki* (nM) of selected inhibitors determined for neuraminidases from different virus subtypes

Neuraminidase source	1	3	4	2	7	8
B/Victoria/504/2000	9,300 ± 500	247 ± 64	1,210 ± 50	665 ± 63	18 ± 2	114 ± 13
A/Panama/2007/99	34,000 ± 4,200	145 ± 6	130 ± 3	209 ± 11	5 ± 1	4 ± 0.2
B/Harbin/7/94	9,400 ± 1,000	296 ± 19	1,430 ± 110	679 ± 66	24 ± 4	135 ± 19
A/Johannes-burg/33/94	36,000 ± 3,400	303 ± 16	1,000 ± 30	247 ± 21	6 ± 0.3	11 ± 1
cHis-tNA full	11,300 ± 1,800	500 ± 50	1,090 ± 90	104 ± 5	19 ± 3	16 ± 2

Example 6

In vitro activity of synthesized compounds (from ketones)

Table 1. *Ki* (nM) of selected inhibitors determined for neuraminidases from different virus subtypes

Neuraminidase source	9	13
B/Victoria/504/2000	232 ± 30	15 ± 1
A/Panama/2007/99	5,8 0,4	10 ± 1
B/Harbin/7/94	209 ± 24	20 ± 2
A/Johannes-burg/33/94	15,7 ± 0,4	12 ± 1
cHis-tNA sol	92 ± 8 nM	2,6 0,2
cHis-tNA full	46 ± 5	1,6 ± 0,1

Example 7

In vivo activity

In influenza the neuraminidase is located at the virus surface. This enzyme is involved in the release of newly synthesized virus particles from the host cell in order to allow the spreading of the infection in the respiratory tract. By inhibition of the neuraminidase the infection cycle of the virus should be stopped. For testing the efficacy of the DCC-generated inhibitors mice were infected with different Influenza strains in the presence or absence of the inhibitors to be tested or the inhibitors were added only after the infection.

In one set of experiments mice (C57BI/6) were either infected intranasal with 200 TCID50 (2.5 LD50) of Influenza A/PR/8/34 (H1N1) or intraperitoneal with 100 TCID50 of A/FPV/ Bratislava (H7N7). The inhibitor HK103 (42nMol) was added at time 0 and again after 24h intranasal or intraperitoneal, respectively. The efficacy of the inhibitor was measured by the survival of animals within a time period of 12 days. In the presence of the inhibitor HK103 all animals survived while in the absence of inhibitor 2/3 (in case of A/FPV/ Bratislava infection)

or all (in case of A/PR/8/34 infection) mice died, indicating equal in vivo efficacy of HK103 against the two different neuraminidase subtype H1N1 and H7N7.

In a second set of experiments the efficacy of the new inhibitor HK103 was compared to the well characterized oseltamivir. Mice (C57Bl/6) were infected intranasal with 200 TCID50 (2.5 LD50) of Influenza A/PR/8/34 (H1N1) either in the presence of the inhibitors (0,1mg/kg body weight) at time point 0 or the inhibitors were applied after infection at 5 consecutive days (day 1 to 5). The efficacy of the inhibitors were measured by the survival of animals within a time period of 21 days. In the complete absence of inhibitor (PBS) or if treated with inhibitor from day 1-5 (HK103 and Oseltamivir) the mice started to die after 7 days. Only 1 (in case of PBS or oseltamivir) or 2 (in case of HK103) mice survived the Influenza infection. In case of infection and direct treatment with the inhibitors (HK103 or oseltamivir) at day 0 all mice survived.

Example 8

Chemical synthesis of compounds

A ***Reductive Amination using aldehydes:***

Methyl (3R, 4R, 5S)-4-Acetamido-3-[(1-(R/S)-cyclohex-3-enyl)methyl]amino-5-[N,N'-Bis-(tert-butyloxycarbonyl)guanidino]-1-cyclohexene-1-carboxylate

28.5 mg (0.057 mmol) (V) were dissolved in 7.5 ml MeOH and 19.5 mg Lindlar's catalyst were added. After hydrogenation for 2 h 30 Min, the reaction mixture was filtered, the catalyst washed with 10 ml MeOH and the solvent evaporated. The crude product was dissolved in 1.5 mL CH_2Cl_2 (dried over 4 A molecular sieves) and 57 µL (0.057 mmol) cyclohex-3-enymethyl aldehyde (1M in CH_2Cl_2), 25 µL (0.17 mmol) triethylamine and 29 µL (0.029 mmol) $ZnCl_2$ (1M in Et_2O) were added. The reaction mixture was then stirred for 16 h at rt. After the addition of 500 µL MeOH and 9.5 mg (0.16 mmol) $NaCNBH_3$, the reaction mixture was stirred for 3 h at rt. The reaction was quenched by the addition of 10 mL sat. $NaHCO_3$/brine (1:1) and extracted with 3 x 10 mL $CHCl_3$. The organic layer was separated, dried over $MgSO_4$ and the solvents evaporated. The remaining residue was purified by chromatography on silica gel using CH_2Cl_2/MeOH (95:5) to afford 25.2 mg (78 %) of the

desired compound (1:1 diastereomeric mixture) as a colorless glass. $_1$H NMR ($CDCl_3$) δ 11.34 (s, 1H), 8.56 (d, 1H, J = 8.3), 7.33 (d, 1H, J = 8.4), 6.81 (s, 1H), 5.62 (s, 2H), 4.30 (ddd, 1H, J = 5.4, 10.5), 3.98-3.86 (m, 2H), 3.74 (s, 3H), 3.38-3.32 (m, 1H), 2.80 (dd, 1H, J = 5.0, 18.0), 2.74-2.68 (m, 1H), 2.40-2.23 (m, 2H), 2.09-1.97 (m, 4H), 1.91 (s, 3H), 1.72-1.64 (m, 2H), 1.47 (s, 18H), 1.26-1.20 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 171.9, 166.8, 163.2, 157.8, 153.1, 140.3, 140.2, 127.6, 127.4, 126.6, 84.3, 80.1, 62.1, 55.7, 53.0, 52.9, 52.4, 49.2, 34.6, 31.4, 30.3, 30.2, 28.6, 28.4, 27.2, 27.1, 25.2, 23.6; LRMS (ESI+) m/z 564 ($[M+H]^+$).

B Reductive Amination using ketones

Methyl (3R, 4R, 5S)-4-Acetamido-3-(1-(R/S)-ethylbutyl)amino-5-(tert-butyloxy-carbonyl)amino-1-cyclohexene-1-carboxylate

32.4 mg (0.09 mmol) **(V)** were reduced with Lindlar's catalyst as described for the synthesis of 16 and the crude product was dissolved in 3 mL MeOH. After the addition of 167 µL (1.35 mmol) 3-hexanone, 45 mg (0.72 mmol) $NaCNBH_3$ and 150 µL acetic acid, the reaction mixture was stirred for 5 h at rt. The reaction was quenched by the addition of 10 mL sat. $NaHCO_3$/brine (1:1) and extracted with 3 x 10 mL $CHCl_3$. The organic layer was separated, dried over $MgSO_4$ and the solvents evaporated. The remaining residue was purified by chromatography on silica gel using CH_2Cl_2/MeOH (95:5) to yield 30 mg (79 %) of the 1:1 diastereomeric mixture of the title compound as a colorless glass.

C Hydrolysis of reductive amination products

(3R, 4R, 5S)-4-Acetamido-3-[(1-(R/S)-cyclohex-3-enyl)methyl]amino-5-guanidinyl-1-cyclohexene-1-carboxylic Acid Hydrochloride (7)

To a solution of 16 mg (0.028 mmol) methyl (3R, 4R, 5S)-4-Acetamido-3-[(1-(R/S)-cyclohex-3-enyl)methyl]amino-5-[N,N'-Bis-(tert-butyloxycarbonyl)guanidino]-1-cyclohexene-1-carboxylate in 600 µL THF was added 300 µL (0.3 mmol) 1 M KOH and the reaction mixture was stirred at rt overnight. The reaction mixture was diluted with 15 mL EtOAc and 1 ml sat. $NaHCO_3$, then carefully acidified with 100 mM HCl to pH ~ 7, 5, 3 and extracted each time with 15 ml EtOAc. The organic extracts were combined, dried over $MgSO_4$ and evaporated to yield the crude free acid. The free acid (13.2 mg, 0.024 mmol) was dissolved in 500 µL $CHCl_3$ and treated with 500 µL $CHCl_3$/TFA (1:1) overnight at rt. The

reaction mixture was evaporated in vacuo and in HV at the oil-pump. Then 1 mL 100 mM HCl was added to the residue and the solvents were evaporated in vacuo and in HV. The remaining glassy material was treated twice with 750 µL CH_3CN, sonicated for 1 Min and the yellow CH_3CN layer removed by syringe. The remainig solid material was dried in HV at the oil-pump. Finally, 500 µL D_2O was added and evaporated in vacuo and in HV to afford 8 mg (73 %) of the title compound (1:1 diastereomeric mixture) as a colorless glass. ^1H NMR (D_2O) δ 6.81 (s. 1H), 5.84-5.68 (m, 2H), 4.45 (dt, 1H, J = 9.8), 4.40-4.31 (m, 1H), 3.98 (ddd, 1H, J = 5.4, 10.5), 3.19-2.94 (m, 3H), 2.57-2.42 (m, 1H), 2.11-2.05 (m, 7H), 1.88-1.75 (m, 2H), 1.40-1.32 (m, 1H); ^{13}C NMR (D_2O) δ 175.8, 168.4, 157.2, 135.0, 129.7, 127.9, 127.8, 125.1, 125.0, 58.6, 50.6, 49.9, 49.2, 49.0, 31.1, 30.4, 28.6, 28.5, 25.7, 25.5, 23.7, 22.4; LRMS (ESI+) m/z 350 ($[M+H]^+$).

D *(3R, 4R, 5S)-4-Acetamido-5-amino-3-(1-(R/S)-ethylbutyl)amino-1-cyclohexene-1-carboxylic Acid Hydrochloride (9)*

(synthesized analogously)

LRMS (ESI+) m/z 298 ($[M+H]^+$).

E *(3R, 4R, 5S)-4-Acetamido-3-(1-(R/S)-ethylbutyl)amino-5-guanidinyl-1-cyclohexene--1-carboxylic Acid Hydrochloride (10)*

(synthesized analogously)

LRMS (ESI+) m/z 340 ($[M+H]^+$).

F *(3R, 4R, 5S)-4-Acetamido-5-amino-3-[1-(R/S)-(1-(R/S)-cyclohex-3-enyl)-propyl]amino-1-cyclohexene-1-carboxylic Acid Hydrochloride (11)*

(synthesized analogously)

LRMS (ESI+) m/z 336 ($[M+H]^+$).

G *(3R, 4R, 5S)-4-Acetamido-3-[1-(R/S)-(1-(R/S)-cyclohex-3-enyl)-propyl]amino-5-guanidinyl-1-cyclohexene-1-carboxylic Acid Hydrochloride (12)*

(synthesized analogously)

LRMS (ESI+) m/z 378 ($[M+H]^+$).

H (3R, 4R, 5S)-4-Acetamido-3-(1-ethylpropyl)amino-5-guanidinyl-1-cyclohexene--1-carboxylic Acid Hydrochloride (13)

(synthesized analogously)

LRMS (ESI+) m/z 326 ($[M+H]^+$).

Example 9

Synthesis of compounds **(14)** and **(15)** containing a dialkylated amino group

The compounds **(14)** and **(15)** were synthesized by methods known as such from **(Y)**.

(3R, 4R, 5S)-4-Acetamido-3-[N-(1R/S)-cyclohex-3-enylmethyl-N'-cyclopropylmethyl]-amino-5-guanidinyl-1-cyclohexene-1-carboxylic Acid Hydrochloride (14)

20 mg (0.035 mmol) (Y) was dissolved in 1.5 mL 1,2-dichloroethane at room temperature and 350 µL (0.35 mmol) cyclopropyl aldehyde (1 M in 1,2-dichloroethane) was added. To the reaction mixture were added 22 mg (0.35 mmol) $NaCNBH_3$ and 150 µL acetic acid. After stirring at rt for 3 h another 22 mg $NaCNBH_3$ and 150 µL acetic acid were added and stirring at rt was continued for 2 h. Then additional 350 µL cyclopropyl aldehyde, 22 mg $NaCNBH_3$ and 500 µL MeOH were added and the reaction mixture was stirred at rt for another 1 h. The reaction was quenched by the addition of 10 mL sat. $NaHCO_3$/brine (1:1) and extracted with 50 ml $CHCl_3$. The organic layer was separated, dried over $MgSO_4$ and evaporated. The remaining residue was purified by chromatography on silica gel using CH_2Cl_2/MeOH (98:2) to yield 15.2 mg (69 %) of the 1:1 diastereomeric mixture of the fully protected compound. Hydrolysis to yield 7.7 mg (61 %) of 24 as a colorless glass followed the representative procedure as described for 17.

LRMS (ESI+) m/z 404 ($[M+H]^+$)

Methyl (3R, 4R, 5S)-4-acetamido-3-[N-(1R/S)-cyclohex-3-enylmethyl-N'-propyl]amino-5-guanidinyl-1-cyclohexene-1-carboxylic Acid Hydrochloride (15)

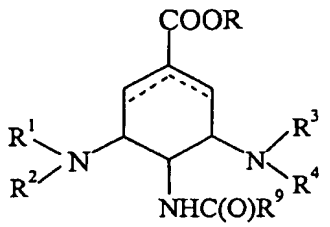
LRMS (ESI+) m/z 392 ([M +H]+)

The compounds showed the following activities:

	Virus particles					Purified NA
IC_{50}	B/Victoria/ 504/2000	B/Harbin/ 7/94	A/Panama/ 2007/99 (H3N2)	A/Johannes -burg/ 33/94 (H3N2)	A/Puerto Rico/8/34 (H1N1)	A/FPV/ Rostock/34
(14)	60 nM	110 nM	125 nM	50 nM	-	80 nM
(15)	45 nM	60 nM	120 nM	32 nM	12 nM	30 nM

Claims

1. A compound according to the formula I



I

wherein

the dotted line denotes a double bond which is present in one of the two possible positions;

R is hydrogen or a C_1-C_4 alkyl group;

R^1 to R^4 are independently of each other selected from the group consisting of:

hydrogen, C_1-C_{20} alkyl groups, C_2-C_{20}-alkenyl groups, C_4-C_{20} aryl groups, C_5-C_{20}-aralkyl groups and C_5-C_{20}-alkaryl groups, all of which groups all can contain one or more hetero atoms from the group consisting of N, O, and S, and which groups can carry one or more substituents from the group consisting of hydroxyl groups and C_1-C_4-alkyl ester groups; or

one of the substituents NR^1R^2 and NR^3R^4 is a guanidino group of the formula $-NR^5-C(NR^6R^7)=NR^8$ in which the substituents R^5 to R^8 independently of each other have the meaning given above for the substituents R^1 to R^4;

R^9 is a C_1-C_4-alkyl group,

or a physiologically acceptable salt or solvate thereof in any stereoisomeric form or mixtures thereof in any ratio,

with the proviso that the compounds in which

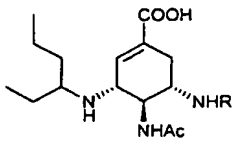
R^1 is H, R^2 is $CH(CH_2CH_3)_2$, R^3, R^4 are H and R is ethyl and

R^1 is H, R^2 is $-CH_2CH_2CH_3$, R^3, R^4 are H and R is ethyl

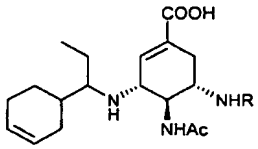
are excluded.

2. A compound according to claim 1, wherein $R^9 = CH_3$, one of the groups NR^1R^2 and NR^3R^4 is -NH-C(NH_2)=NH and the other group is -NH_2 which is substituted by an alkyl group which is optionally substituted by a 4- to 6-membered carbocycle optionally carrying 1 to 3 olefinic bonds.

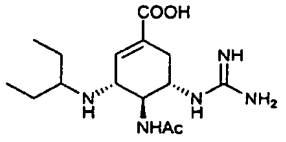
3. A compound according to claim 1 which is selected from the following compounds in any stereoisomeric form:



9 R = H
10 R = C(=NH)NH_2



11 R = H
12 R = C(=NH)NH_2



13

4. As a compound according to claim 1, 4-acetamido-3-[(1-cyclohex-3-enyl)methyl]amino-5-guanidinyl-1-cyclohexene-1-carboxylic acid.

5. As a compound according to claim 1, 4-acetamido-3-[N-cyclohex-3-enylmethyl-N'-cyclopropylmethyl]-amino-5-guanidinyl-1-cyclohexene-1-carboxylic acid or methyl 4-acetamido-3-[N-cyclohex-3-enylmethyl-N'-propyl]amino-5-gua-nidinyl-1-cyclohexene-1-carboxylic acid in any stereoisomeric form.

6. A method of treating the activity of neuraminidase, preferably influenza neuraminidase, in a mammal, preferably a human, which method comprises administering an effective amount of a compound according to formula I as defined in claim 1 to the said mammal.

7. A compound according to formula I as defined in claim 1 for use as a medicament.

8. The use of a compound according to formula I as defined in claim 1 for the manufacture of a medicament for the inhibition of neuraminidase, in particular for the inhibition of influenza neuraminidase.

9. A medicament which contains an effective amount of a compound of formula I as defined in claim 1, preferably for inhibiting the activity of neuraminidase, in particular for inhibiting the activity of influenza neuraminidase, which medicament comprises an

effective dose of a compound of the formula I or a physiologically acceptable salt or a physiologically acceptable solvate thereof and a pharmaceutically acceptable carrier.

10. A method of forming a library of components which are potentially capable of binding to neuraminidase, in particular influenza neuraminidase, which method comprises

i) selecting a plurality of molecules carrying a functionality which may interact with a binding site of neuraminidase, said molecules furthermore having a linking group which is capable of interacting with other linking groups under the formation of reversible bonds;

ii) reacting the molecules carrying the functionality with a molecule according to formula I as defined in claim 1 in the presence of the target, under conditions where a formation of reversible bonds between the linking groups on the molecule I and on the molecules carrying a functionality occurs.

11. The method according to claim 10, wherein, before the target is added in step ii), a step i)a) is conducted in which the molecule I and the molecules carrying the functionality are reacted in the absence of the target until equilibrium is reached.

12. The method according to claim 10 or 11, wherein the molecule I is biologically inactive.

13. The method according to claim 10 or 11, wherein the molecule I is biologically active.

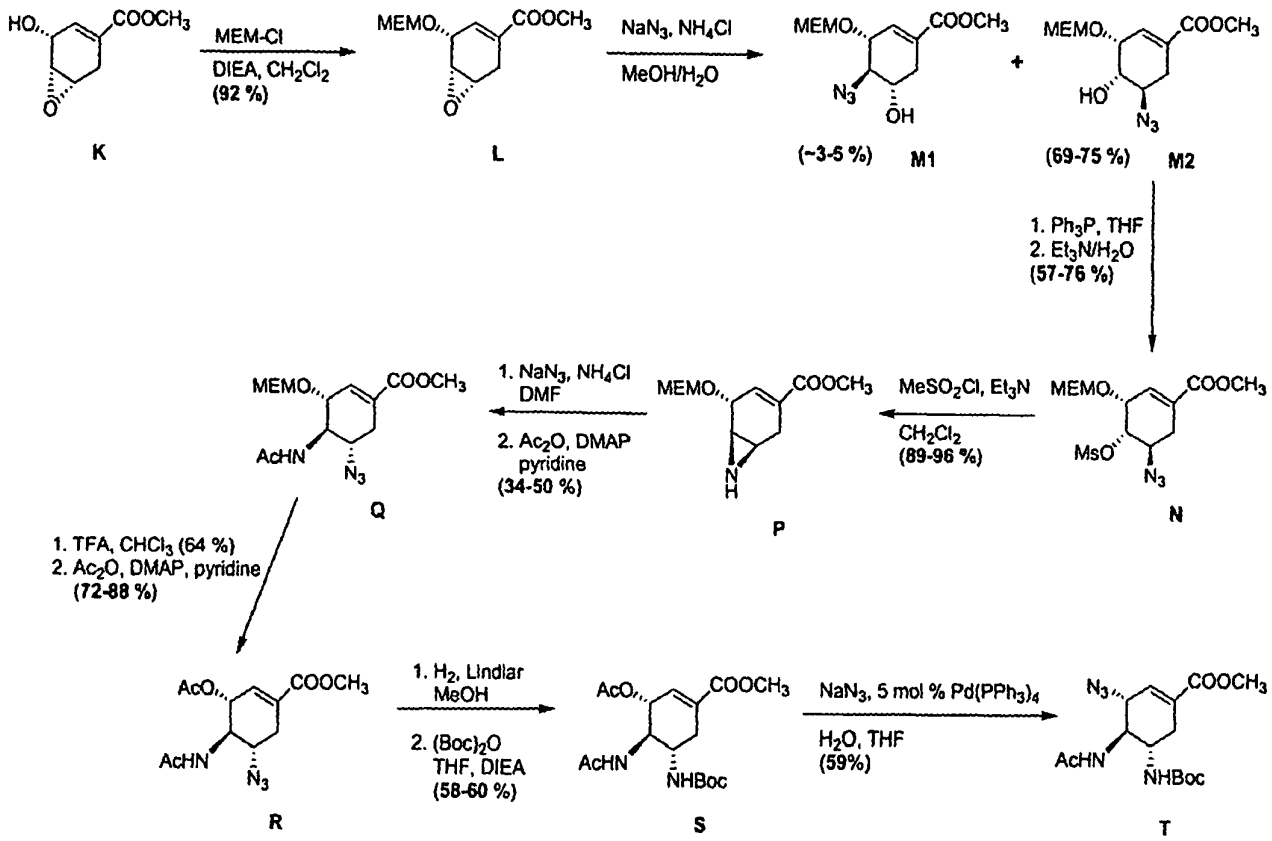
14. The method according to any of claims 10 to 13, wherein the reversible bonds formed between the linking groups present on the molecule I and on the molecules carrying the functionality are covalent bonds.

15. A dynamic combinatorial library which is obtainable by the method according to any of claims 10 to 14.

16. A method of assessing the binding capacity of a component to bind to neuraminidase, which method comprises carrying out the method as described in any of the claims 10 to 15, and which method additionally comprises

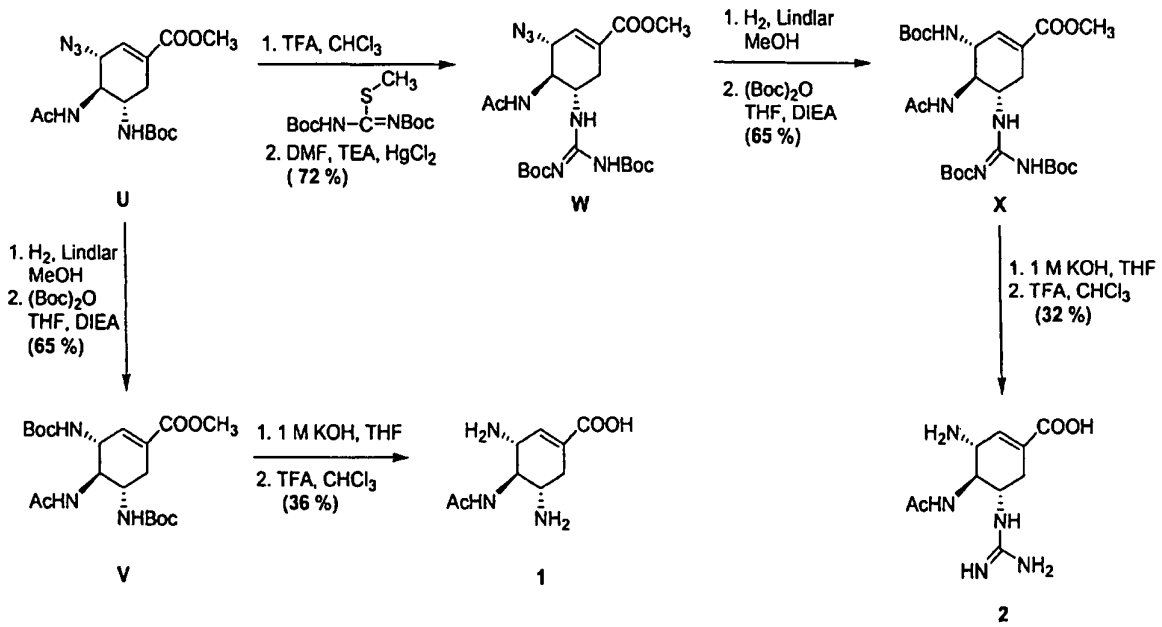
iii) identifying the components which preferably bind to the target

17. The method according to claim 16, wherein the compounds are identified by analyzing the reaction mixture and comparing the results obtained with the results obtained by analyzing a reaction mixture obtained under the same conditions from the same starting materials, but in the absence of the neuraminidase.



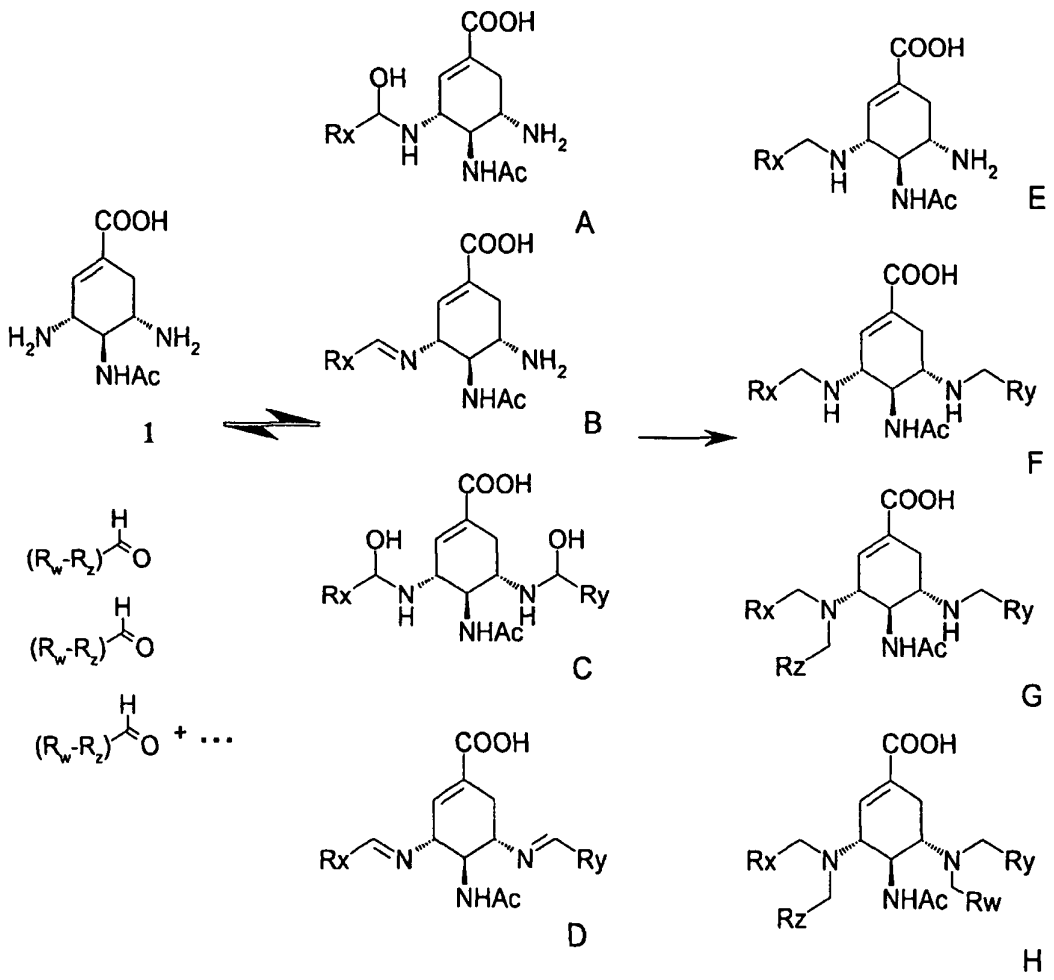
Kim C.C. *et al.*, *J. Am.Chem.Soc.* **1997**, *119*, 681-690
Corey E. J. *et al. Synthesis* **1975**, 590-591
Lew W. *et al.*, *Bioorg. Med. Chem Lett.* **1998**, *8*, 3321-3324

Scheme 1: Synthesis of the compounds (part A)



Corey E. J. *et al. Synthesis* **1975**, 590-591
Kim K. S. *et al. Tetrahedron Lett.* **1993**, *34*, 7677-7680
Baker T. J. *et al. Synthesis* **1999**, 1423-1426

Scheme 1: Synthesis of the compounds **1** and **2** (part 2)



R_w - R_z ≡ substituents with the meanings defined for R^1 - R^4 in formula I

Scheme 2: Generation of a Dynamic Combinatorial Library with the scaffold

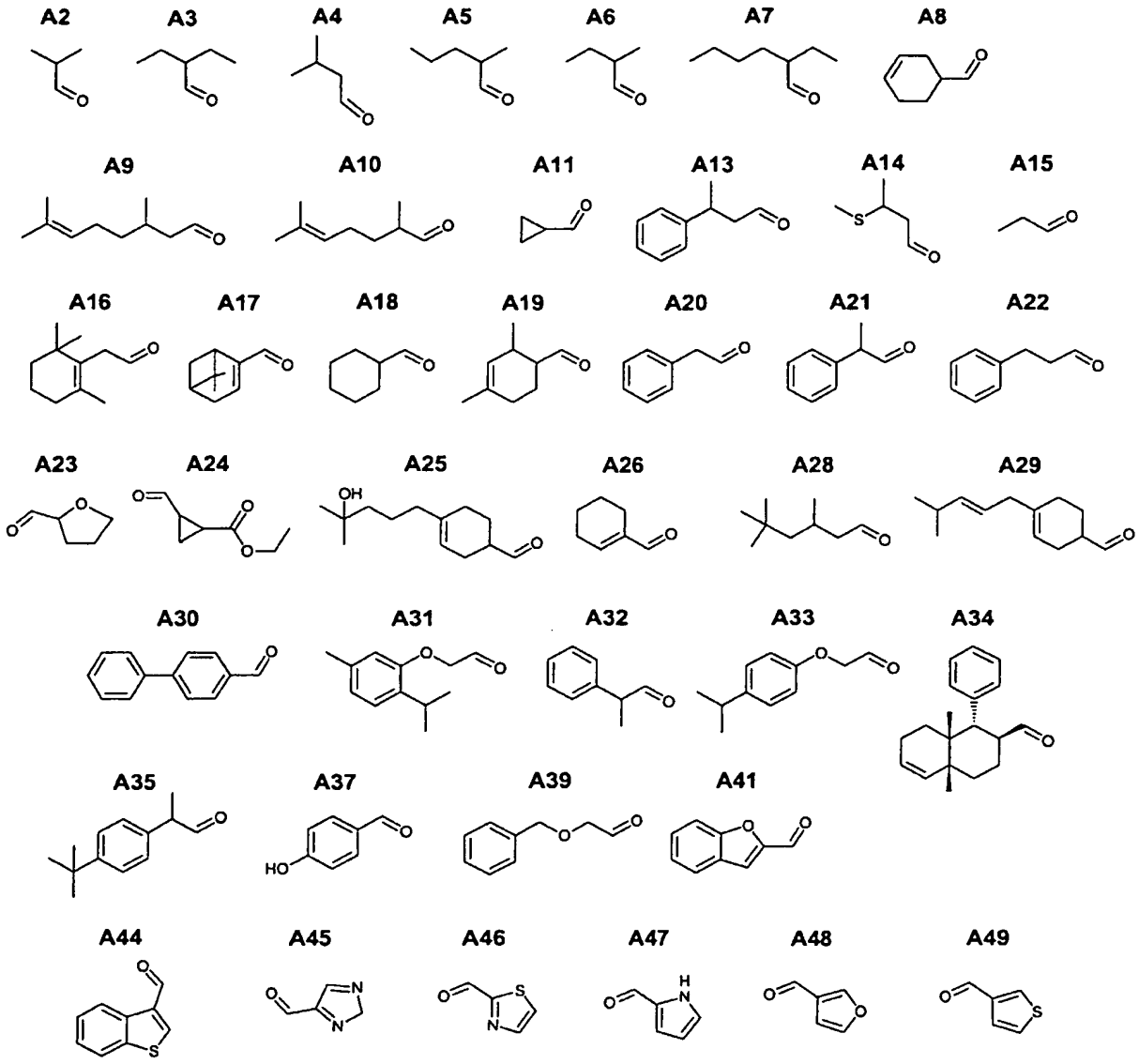


Fig. 1 aldehydes used in the generation of dynamic combinatorial libraries

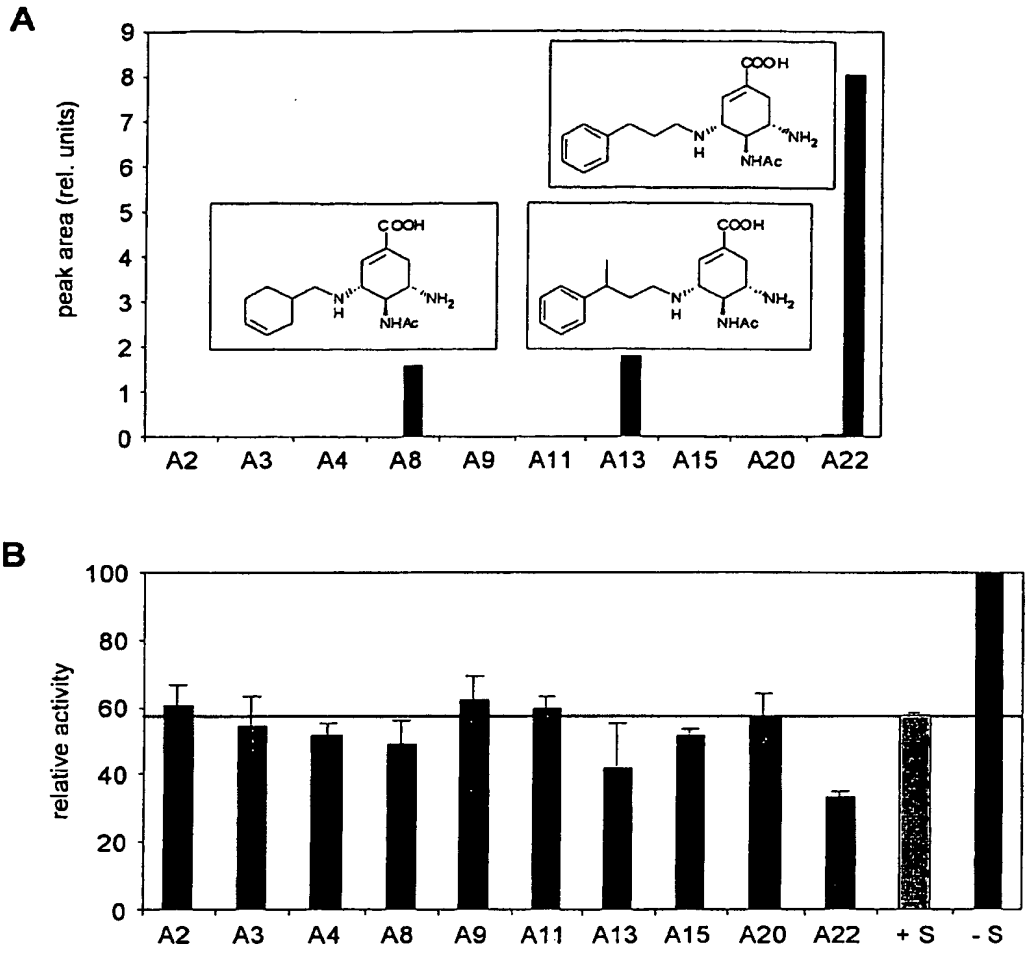


Figure 2: HPLC-MS-Analysis of the library generated with scaffold **4**

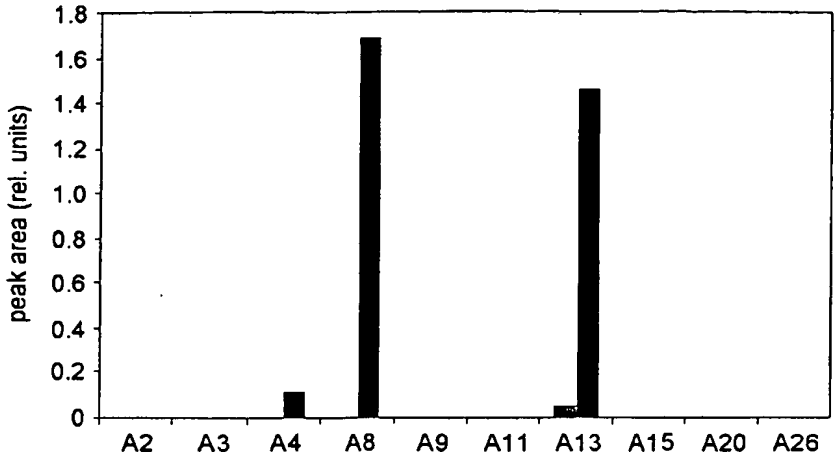


Figure 3: HPLC-MS-analysis of a library generated in the absence of aldehyde 22

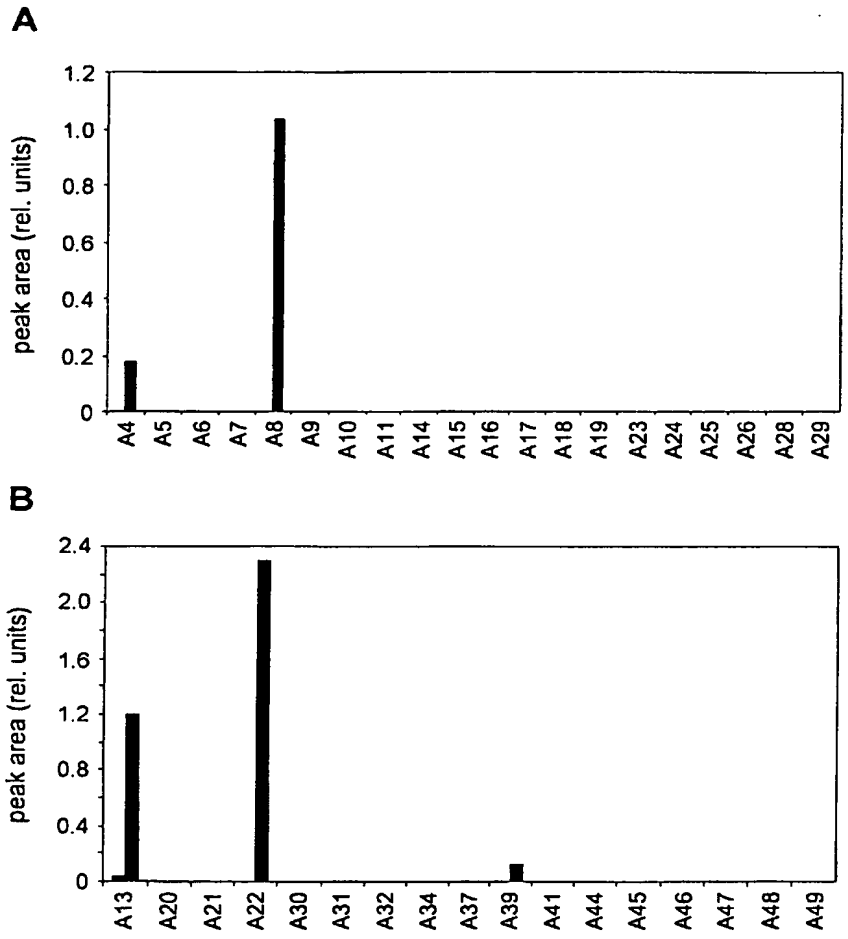


Figure 4: HPLC-MS-analysis of libraries generated with subgroups of the aldehydes originally employed

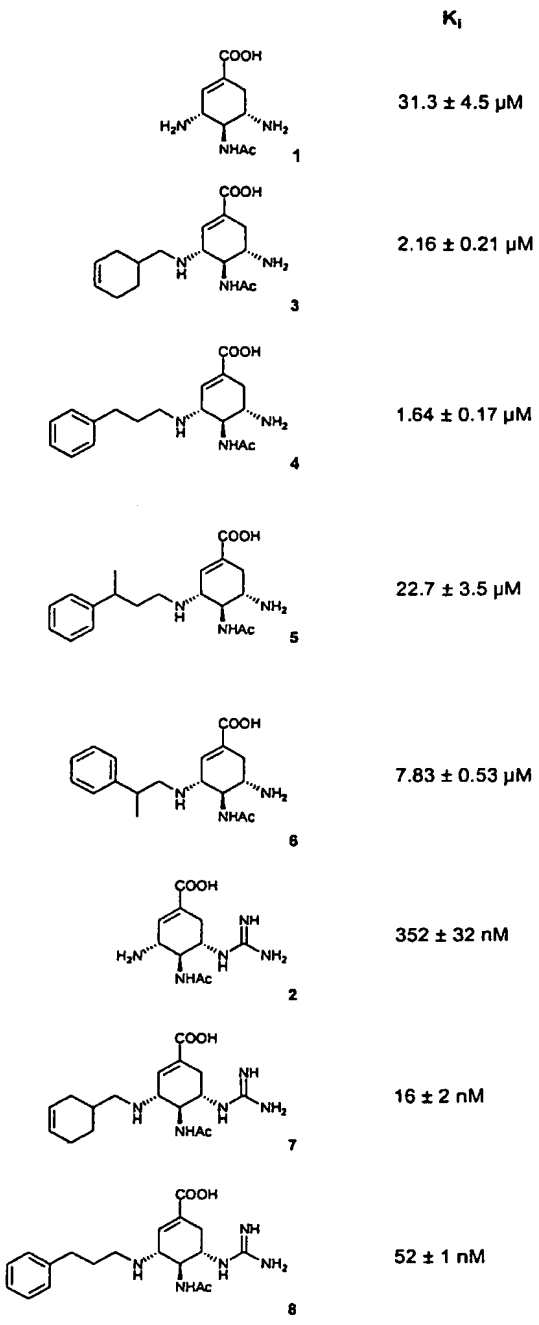


Fig. 5: Ki-values of compounds identified by DCC-screening